DIVISION - CONTINUATION - CONTINUATION-IN-PART APPLICATION TRANSMITTAL FORM			Attorney Docket No.: A-345C
	Anticipated Classification Of This Application: Class 435	Subclass	Prior Application: Examiner G. Draper — Art Unit 1646

To the Assistant Commissioner for Patents:

This is a request for filing a ☒ continuation ☐ divisional ☐ continuation-in-part application, under 37 CFR 1.53(b), of pending prior application Serial No. 08/920,608 filed on August 27, 19 97, of Pelleymounter, et al. for OB PROTEIN COMPOSITIONS AND METHODS

For CONTINUATION or DIVISIONAL APPLNs only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 1b, below, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

1. ☒ Transmitted herewith are:
   - ☒ 27 pages of specification, 2 pages of claim(s) and 1 page of abstract, **totaling** 30 pages.
   - ☐ ____ sheet(s) of drawings.
   - ☒ 2 pages of Oath or Declaration by the applicant(s):
     - ☐ a. Newly executed (original or copy)
     - ☒ b. Copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional applns. only)*
   - ☒ 4 pages of Sequence Listing; sequence statement.

2. ☒ The filing fee is calculated below:

For	Number Filed		Number Extra	Rate	Fee
Total Claims	12	- 20 =	0	x $18.00 =	$ 0.00
Independent Claims	4	- 3 =	1	x $78.00 =	78.00
Multiple Dependent Claims	0			+ $260.00 =	0.00
Basic Fee				$760.00 =	760.00
				Total Filing Fee	$ 838.00

3. ☒ Please charge Deposit Account No. 01-0519, in the name of Amgen Inc., in the amount of $ 838.00. An original and one copy are enclosed.

4. ☒ Throughout the prosecution of this application, if any extension of time is necessary, please consider this a request therefor.

5. ☒ The Commissioner is hereby authorized to charge any additional filing fees which may be required by the accompanying application, any additional fees which may be required during pendency of this application as required by 37 CFR 1.16 or 1.17, or credit any overpayment to Deposit Account No. 01-0519 throughout the prosecution of this application.

6. ☐ Cancel in this application original claims ____________ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

EXPRESS MAIL CERTIFICATE

"Express Mail" mail labeling number EL198797275US Date of Deposit. August 2, 1999

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1 10 on the date indicated above and is addressed to Box Patent Application, Assistant Commissioner for Patents, Washington, DC 20231

Freddie Craft — F Craft
Printed Name — Signature

7. ☒ **Preliminarily, please amend the specification by inserting before the first line the following:**

-- This application is a ☒ continuation ☐ division of application Serial No. -08/920,608, filed August 27, 1997; which is a continuation of 08/474,833, filed June 7, 1995, now abandoned, which are hereby incorporated by reference.--

8. ☐ Transfer the drawings from the prior application to this application and abandon said prior application as of the filing date accorded this application. A duplicate copy of this sheet is enclosed for filing in the prior application file. (May only be used if signed by person authorized by § 1.138 and before payment of base issue fee.)

8a. ☐ New formal drawings are enclosed.

9. ☐ Priority of application Serial No. ______________ filed on ______________ in ______________ (country) is claimed under 35 USC 119.

9a. ☐ The certified copy has been filed in prior application Serial No. ______________ filed ______________

10. ☒ The prior application is assigned of record to Amgen Inc.

11. ☒ A preliminary amendment is enclosed.

12. ☒ Also enclosed Associate Power of Attorney (copy)

13. ☐ Other: ______________

14. ☒ The power of attorney in the prior application is to:
Ron K. Levy, Registration No. 31,539; Steven M. Odre, Registration No. 29,094;
Karol M. Pessin, Registration No. 34,899, Joan D. Eggert, Registration No. 32,980

a. ☐ The power appears in the original papers in the prior application.

b. ☒ Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.

c. ☒ Address all future communications to
Joan D. Eggert
at the address below.

Signator: ☐ Assignee of complete interest

☒ Attorney of record

Karol M. Pessin
Attorney for Applicants
Registration No. 34,899
Phone: (805) 447-2193
Date: August 2, 1999

Please send all future correspondence to:

U. S. Patent Operations/ JDE
Dept. 430, M/S 27-4-A
AMGEN, INC.
One Amgen Center Drive
Thousand Oaks, California 91320-1799

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: PELLEYMOUNTER, et al.

Serial No.: Not Yet Received

Group Art Unit No.: Not Yet Assigned

Filed: August 2, 1999

Examiner: Not Yet Assigned

For: OB PROTEIN COMPOSITIONS AND METHODS

Docket No.: A-345C

PRELIMINARY AMENDMENT UNDER 37 CFR 1.121

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Please amend the accompanying application under 37 CFR 1.12 as follows:

In the Specification

Please amend the specification by inserting as the first paragraph the following:

--This application is a continuation of application Serial No. 08/920,608, filed August 27, 1997; which is a continuation of 08/474,833, filed June 7, 1995, now abandoned, which are hereby incorporated by reference.--

At page 3, line 29: after "SEQ. ID. Nos." delete --2 and 4-- and insert --3 and 6--

At page 11, line 17: delete "SEQ. ID. No. 2"

At page 11, line 19: delete "SEQ. ID. No. 1"

At page 12, line 4: after "3," delete --and 4-- and insert --4, 5, and 6--

At page 12, line 8: after "SEQ. ID. No." delete --3-- and insert --4--

At page 13, line 7: delete "SEQ. ID. No. 1 or 2"

At page 13, line 9: delete "SEQ. ID. No. 1"

At page 16, line 2: after "SEQ. ID. Nos." delete --1 and 2-- and insert --1, 2 and 3--

At page 25, line 2: after "SEQ. ID. Nos." delete --3 and 4-- and insert --4, 5 and 6--

EXPRESS MAIL CERTIFICATE

"Express Mail" mail labeling number EL198797275US   Date of Deposit August 2, 1999

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1 10 on the date indicated above and is addressed to Box Patent Application, Assistant Commissioner for Patents, Washington, DC 20231

Freddie Craft
Printed Name

Signature

In the Claims

1. (Once amended) A method of treating excess weight in a mammal by continuous administration of 1 mg protein/kg body weight/day or less of an OB protein selected from the group consisting of:

(a) recombinant methionyl murine OB protein [SEQ. ID. No. 2)];
(b) recombinant methionyl human OB protein [(SEQ ID No. 1)];
(c) the protein of (a) or (b) lacking the methionyl residue at position -1;
(d) the protein of (a), (b) or (c) lacking a glutamine at position 28; and
(e) a chemically modified derivative of (a), (b),(c) or (d).

8. (Once amended) A DNA sequence according to SEQ ID No. [3] 4.

11. (Once amended) A method of refolding partially purified OB protein in a solution obtained from inclusion bodies, said partially purified OB protein selected from the group consisting of:

(a) recombinant methionyl murine OB protein (SEQ. ID. No. [2] 3);
(b) recombinant methionyl human OB protein (SEQ ID No. [1] 6);
(c) the protein of (a) or (b) lacking the methionyl residue at position -1;
wherein said refolding is accomplished using N-lauroyl sarcosine.

Please charge any fees associated herewith to Deposit Account 01-0519 and consider this a petition therefore if appropriate.

Respectfully submitted,

Karol M. Pessin
Attorney for Applicants
Registration No.: 34,899
Phone: (805) 447-2193
Date: August 2, 1999

Please send all future correspondence to:

US Patent Operations/JDE
Dept. 430, M/S 27-4-A
AMGEN INC.
One Amgen Center Drive
Thousand Oaks, California 91320-1799

OB PROTEIN COMPOSITIONS AND METHOD

Field of the Invention

The present invention relates to OB protein compositions and methods for preparation and use thereof.

Background

Although the molecular basis for obesity is largely unknown, the identification of the "OB gene" and protein encoded by ("OB protein") has shed some light on mechanisms the body uses to regulate body fat deposition. Zhang et al., Nature 372: 425-432 (1994); see also, the Correction at Nature 374: 479 (1995). The OB protein has been demonstrated to be active *in vivo* in both ob/ob mutant mice (mice obese due to a defect in the production of the OB gene product) as well as in normal, wild type mice. The biological activity manifests itself in, among other things, weight loss. To date, however, optimum conditions for obtaining the rapid weight loss in normal animals has not been ascertained. In fact, some studies have shown that, when administered by injection, rather large dosages (10 mg of recombinant murine protein/kg body weight/day) are necessary for normal mice to lose 2.6% of their body weight (at the end of a 32 day period). While presently uncertain, one explanation for the necessity of such large dosages is that the optimum weight loss effects are seen predominantly when the protein is in constant circulation, a condition that may not be efficiently achieved by injecting the protein.

Summary of the Invention

The present invention stems from the observation that, as compared to administering OB protein by injection, administering OB protein by continuous pump infusion results in equivalent (or better) weight loss, in a shorter time, and with substantially lower dosages. The working example below demonstrates that a dose of 0.5 mg protein/kg body weight/day, administered via implantable osmotic pump, results in a weight loss of over 4% (as compared to baseline weight). This is in substantial contrast to other studies where similar, or less weight loss (at a comparable time point) was observed with intraperitoneal injection at the relatively high dosage of 10 mg of protein/kg body weight/day.

Thus, one aspect of the present invention is a method of treating excess weight by administering OB protein in a form for constant supply, at a dosage of less than or equal to about 1 mg protein/kg body weight/day. The dosage of less than or equal to about 1 mg protein/kg/day refers to dosages sufficient to result in observable weight loss. This is apparent from the present studies where a dosage of 0.5 mg/kg/day was sufficient to result in observable weight loss when continuously administered. In studies where injection had been the mode of administration, far higher dosages were required for weight loss. At injection dosages of 0.1 and 1 mg/kg/day, substantially no weight loss was observed in wild type (normal) mice. For example, in one study, at a comparable time point (6th day), there was a .2% loss at the 1 mg/kg dose (data not shown). Minimal weight loss was observed at the relatively high 10 mg/kg/day dose. (1.9% weight loss at day 6, data not shown). Thus, the present invention provides for dosages of 1 mg/kg/day or less

when administered so that the supply of protein is continuous.

Connected with the present studies are the compositions and methods used for production of recombinant murine and human OB protein. The first example below discloses the preparation of recombinant murine protein, and the second example below discloses the preparation of recombinant human protein.

Additional aspects of the present invention, therefore, include the below compositions and methods for preparing recombinant murine methionyl OB protein and recombinant human methionyl OB protein, including DNA sequences, vectors, host cells, methods of fermentation, and methods of purification.

<ins>Detailed Description</ins>

The present invention stems from the observation that continuous administration of OB protein results in the need for much lower dosages for weight loss than those dosages required by acute daily injection. As set forth above, a dosage of 1 mg protein/kg body weight/day or less, continuously administered, resulted in rapid weight loss. When the underivatized protein was administered by acute injection at the 1 mg/kg/day dose, almost no weight loss in wild type (normal) mice.

The OB protein may be selected from the recombinant murine and human methionyl proteins set forth below (SEQ. ID Nos. 2 and 4) or those lacking a glutaminyl residue at position 28. (<ins>See</ins> Zhang et al, Nature, <ins>supra</ins>, at page 428.) The recombinant human OB gene product is, as a mature protein, 146 amino acids; some of the DNAs obtained were observed to encode a protein lacking a glutamine residue at position 28. Zhang et al., Nature <ins>372</ins> at 428. The murine protein is substantially homologous to the human protein,

particularly as a mature protein, and, further, particularly at the N-terminus. One may prepare an analog of the recombinant human protein by altering (such as substituting amino acid residues), in the recombinant human sequence, the amino acids which diverge from the murine sequence. Because the recombinant human protein has biological activity in mice, such analog would likely be active. Proteins lacking an N-terminal methionyl residue, such as those produced by eukaryotic expression, are also available for use.

In addition, although the present working example involved continuous administration via implantable pump, it is contemplated that other modes of continuous administration may be practiced. For example, chemical derivatization may result in sustained release forms of the protein which have the effect of continuous presence in the blood stream, in predictable amounts. Thus, one may derivatize the above proteins to effectuate such continuous administration. The dosage of 1 mg protein/kg body weight/day or less herein refers to the mass of protein, exclusive of other chemical moieties used to derivatize the protein.

Generally, the present protein (herein the term "protein" is used to include "peptide", unless otherwise indicated) may be derivatized by the attachment of one or more chemical moieties to the protein moiety. The chemically modified derivatives may be further formulated for intraarterial, intraperitoneal, intramuscular subcutaneous, intravenous, oral, nasal, pulmonary, topical or other routes of administration. Chemical modification of biologically active proteins has been found to provide additional advantages under certain circumstances, such as increasing the stability and circulation time of the

therapeutic protein and decreasing immunogenicity. See U.S. Patent No. 4,179,337, Davis et al., issued December 18, 1979. For a review, see Abuchowski et al., in Enzymes as Drugs. (J.S. Holcerberg and J. Roberts, eds. pp. 367-383 (1981)). A review article describing protein modification and fusion proteins is Francis, Focus on Growth Factors 3: 4-10 (May 1992) (published by Mediscript, Mountview Court, Friern Barnet Lane, London N20, OLD, UK). For the present continuous administration, it is preferred that the chemical modification allow for an increase in circulation time of the protein, so that a dosage of about 1 mg protein (exclusive of chemical moiety)/kg body weight of a mammal/day or less will result in weight loss of a mammal. The present continuous administration will provide for weight loss of approximately 5% of body mass in a period of 7 or fewer days.

The chemical moieties suitable for derivatization may be selected from among water soluble polymers. The polymer selected should be water soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis, and other considerations. For the present proteins and peptides, the effectiveness of the derivatization may be ascertained by administering the derivative, in the desired form (i.e., by osmotic pump, or, more preferably, by injection or infusion, or,

further formulated for oral, pulmonary or nasal delivery, for example), and measuring weight loss.

The water soluble polymer may be selected from the group consisting of, for example, polyethylene glycol, copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols and polyvinyl alcohol. Polyethylene glycol propionaldenhyde may have advantages in manufacturing due to its stability in water.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 2kDa and about 100kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog).

The number of polymer molecules so attached may vary, and one skilled in the art will be able to ascertain the effect on function. One may mono-derivatize, or may provide for a di-, tri-, tetra- or some combination of derivatization, with the same or different chemical moieties (e.g., polymers, such as

different weights of polyethylene glycols). The proportion of polymer molecules to protein (or peptide) molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio (in terms of efficiency of reaction in that there is no excess unreacted protein or polymer) will be determined by factors such as the desired degree of derivatization (e.g., mono, di-, tri-, etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched, and the reaction conditions.

The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art. E.g., EP 0 401 384 herein incorporated by reference (coupling PEG to G-CSF), see also Malik et al., Exp. Hematol. 20: 1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residue. Those having a free carboxyl group may include aspartic acid residues, glutamic acid residues, and the C-terminal amino acid residue. Sulfhydrl groups may also be used as a reactive group for attaching the polyethylene glycol molecule(s). Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group. Attachment at residues important for receptor binding should be avoided if receptor binding is desired.

One may specifically desire N-terminally chemically modified protein. Using polyethylene glycol as an illustration of the present compositions, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective N-terminal chemical modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved. For example, one may selectively N-terminally pegylate the protein by performing the reaction at a pH which allows one to take advantage of the pK_a differences between the ε-amino group of the lysine residues and that of the α-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. Using reductive alkylation, the water soluble polymer may be of the type described above, and should have a single reactive aldehyde for coupling to

the protein. Polyethylene glycol propionaldehyde, containing a single reactive aldehyde, may be used.

In yet another aspect of the present invention, provided are methods of using pharmaceutical compositions of the proteins and derivatives. Such pharmaceutical compositions may be for administration for injection, or for oral, pulmonary, nasal or other forms of administration which allow for the desired circulating dose of about 1 mg protein/kg body weight/day or less. In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of protein or derivative products of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimersol, benzyl alcohol) and bulking substances (e.g., lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hylauronic acid may also be used, and this may have the effect of promoting sustained duration in the circulation. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present proteins and derivatives. *See*, *e.g*., Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by reference. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form. The effective amounts are those herein described.

The OB proteins and derivatives described are useful for modulation of the rate or quantity of fat cell deposition in a mammal. This is thought to be accomplished, in part, by a reduction in appetite, i.e., a reduction in food intake. Thus, one observable result is weight loss, or, put another way, a method of treating excess weight (via weight loss). Thus, the present compositions are useful for the manufacture of a medicament for treating excess weight in a mammal. Another aspect is a method for reducing appetite. Either of these aspects, modulation of fat deposition or modulation of appetite, are particularly important treatments for humans (or other mammals) who desire to lose weight.

One skilled in the art will be able to ascertain other effective dosages by administration and observing weight loss. Here, the dosage of 1 mg protein/kg body weight/day or less was seen to be particularly effective, when administered on a continuous basis. More particularly, the dosage of 0.5 mg/kg body weight/day was seen to be particularly effective on normal mice. Excess weight refers to body mass for which removal is desired. It is contemplated that the present compositions and methods will be used to treat cases where removal of such excess weight (as a result of the present invention) will benefit other health concerns, such as diabetes, high blood pressure or cardiac problems, high cholesterol levels, low locomotion levels and other manifestations of excess weight. As such, the present compositions and methods may be used in conjunction with other medicaments, such as those useful for the treatment of diabetes (e.g., insulin, and possibly amylin), cholesterol and blood pressure lowering medicaments, and locomotion increasing medicaments (e.g., amphetamines). Such

administration may be simultaneous or may be in serriatim.

In addition, the present compositions and methods may be used in conjunction with surgical procedures, such as cosmetic surgeries designed to alter the overall appearance of a body (e.g., liposuction or laser surgeries designed to reduce body mass). The health benefits of cardiac surgeries may be increased with concomitant use of the present compositions and methods.

Therefore, the present invention encompasses a method of treating excess weight in a mammal by continuous administration of 1 mg protein/kg body weight/day or less of an OB protein selected from the group consisting of:

(a) recombinant methionyl murine OB protein (SEQ. ID. No. 2);

(b) recombinant methionyl human OB protein (SEQ ID No. 1);

(c) the protein of (a) or (b) lacking the methionyl residue at position -1;

(d) the protein of (a), (b) or (c) lacking a glutamine at position 28; and

(e) a chemically modified derivative of (a), (b),(c) or (d), wherein the chemical modification allows for an increase in circulation time.

Preferably, the composition of subpart (e) is a pegylated derivative, and, more preferably, an N-terminally pegylated derivative.

The derivative of subpart (e) allows for continuous administration of the protein by increasing the circulation time of the (unmodified) protein. The present invention also encompasses a method of treating excess weight where the method of continuous administration is by implantable pump, such as an osmotic pump.

In other aspects, the present invention relates to recombinant murine and recombinant human OB DNAs and proteins, such as those of SEQ. ID NOs. 1, 2, 3, and 4, below. The recombinant proteins below are bacterially expressed, and contain N-terminal methionyl residues. Vectors and host cells useful for producing such proteins are also provided. The vectors include pCFM1656 containing SEQ ID No. 1 or 3, and host cells containing such vectors.

Methods for preparation of the recombinant proteins are also provided, including methods for fermentation and methods for purification.

In particular, the use of sarcosine for refolding of OB protein in solution, obtained from bacterial inclusion bodies, provided for extremely efficient refolding. When proteins are expressed in bacteria, they may not be in the proper three-dimensional configuration, or, as referred to herein, properly refolded. The three dimensional configuration may be critical for biological activity, and storage stability. Although Sarckosyl has been used in processes for purification of another protein (G-CSF, e.g., WO 89/10932), surprisingly, the use of sarcosine for the OB protein has resulted in a refolding efficiency of over 95%. Contemplated herein is the use of N-lauroylsarcosine in a range of 0.5% - 2.0 % weight per volume of OB protein in solution (obtained from inclusion bodies). With the use of 1% sodium sarcosine, the refolding efficiency, as determined by SDS PAGE and reverse phase HPLC, was 95% or greater. While one skilled in the art will recognize that other compositions may be used for refolding, the use of N-lauroyl sarcosine, as illustrated in the examples below, is particularly advantageous for providing extremely efficient refolding. The removal of sarcosine was accomplished using Dowex®.

Therefore, the present invention also includes a method of refolding partially purified OB protein in a solution obtained from inclusion bodies, said partially purified OB protein selected from the group consisting of:

(a) recombinant methionyl murine OB protein (SEQ. ID. No. 2);

(b) recombinant methionyl human OB protein (SEQ ID No. 1);

(c) the protein of (a) or (b) lacking the methionyl residue at position -1;

wherein said refolding is accomplished using sarcosine.

The present invention also includes methods of wherein said N-lauroyl sarcosine is used at a concentration of 0.5% - 2.0% weight per volume of solution, and, more particularly, the use of 1% N-lauroyl sarcosine. An oxidizing agent, such as copper sulfate, is also used in the refolding process.

The following examples are offered to more fully illustrate the invention, but are not to be construed as limiting the scope thereof.

EXAMPLE 1: Use of Murine OB Protein in a Continuous Pump Infusion System

This example demonstrates that continuous infusion of OB protein results in weight loss in normal mice. Normal (non-obese) mice were administered murine OB protein via osmotic pump infusion. A dosage of 0.5 mg protein/kg body weight/day resulted in a 4.62% (+/- 1.34%) loss from baseline weight by the 6th day of infusion.

MATERIALS AND METHODS

Animals: Wild type (+/+) C57B16 mice were used for this experiment. The age of the mice at the initial time point was 8 weeks, and the animals were weight stabilized. 10 mice were used for each cohort (vehicle vs. protein).

Animal Handling.

Feeding and weight measurement. Mice were given ground rodent chow (PMI Feeds, Inc.) in powdered food feeders (Allentown Caging and Equipment) which allowed a more accurate and sensitive measurement than use of regular block chow. Weight was measured at the same time each day (2:00 p.m.), for a period of 6 days. Body weight on the day prior to the infusion was defined as baseline weight. The mice used weighed 18-22 grams.

Housing. Mice were single-housed, and maintained under humane conditions.

Administration of Protein or Vehicle. Protein (as described below) or vehicle (phosphate buffered saline, pH 7.4) were administered by osmotic pump infusion. Alzet osmotic minipumps (Alza, Palo Alto, CA, model no. 1007D) were surgically placed in each mice in a subcutaneous pocket in the subscapular area . The pumps were calibrated to administer 0.5 μl protein in solution per hour for a dosage of 0.5 mg protein/kg body weight/day.

Controls: Control animals were those who had a Alzet osmotic minipump infusing phosphate buffered saline (pH 7.4).

Protein: Recombinant murine OB protein was used for the present experiments, generally at a concentration of about 0.9 mg/ml phosphate buffered saline, pH 7.4. The amino acid sequence (and DNA sequence) used was the following:

<u>Recombinant murine met OB (double stranded) DNA and amino acid sequence (</u>Seq. ID. Nos. 1 and 2):

```
    TCTAGATTTGAGTTTTAACTTTTAGAAGGAGGAATAACATATGGTACCGATCCAGAAAGT
  9 -+---------+---------+---------+---------+---------+--------- 68
    AGATCTAAACTCAAAATTGAAAATCTTCCTCCTTATTGTATACCATGGCTAGGTCTTTCA
                                              M  V  P  I  Q  K  V  -

    TCAGGACGACACCAAAACCTTAATTAAAACGATCGTTACGCGTATCAACGACATCAGTCA
 69 -+---------+---------+---------+---------+---------+--------- 128
    AGTCCTGCTGTGGTTTTGGAATTAATTTTGCTAGCAATGCGCATAGTTGCTGTAGTCAGT
     Q  D  D  T  K  T  L  I  K  T  I  V  T  R  I  N  D  I  S  H  -

    CACCCAGTCGGTCTCCGCTAAACAGCGTGTTACCGGTCTGGACTTCATCCCGGGTCTGCA
129 -+---------+---------+---------+---------+---------+--------- 188
    GTGGGTCAGCCAGAGGCGATTTGTCGCACAATGGCCAGACCTGAAGTAGGGCCCAGACGT
     T  Q  S  V  S  A  K  Q  R  V  T  G  L  D  F  I  P  G  L  H  -

    CCCGATCCTAAGCTTGTCCAAAATGGACCAGACCCTGGCTGTATACCAGCAGGTGTTAAC
189 -+---------+---------+---------+---------+---------+--------- 248
    GGGCTAGGATTCGAACAGGTTTTACCTGGTCTGGGACCGACATATGGTCGTCCACAATTG
     P  I  L  S  L  S  K  M  D  Q  T  L  A  V  Y  Q  Q  V  L  T  -

    CTCCCTGCCGTCCCAGAACGTTCTTCAGATCGCTAACGACCTCGAGAACCTTCGCGACCT
249 -+---------+---------+---------+---------+---------+--------- 308
    GAGGGACGGCAGGGTCTTGCAAGAAGTCTAGCGATTGCTGGAGCTCTTGGAAGCGCTGGA
     S  L  P  S  Q  N  V  L  Q  I  A  N  D  L  E  N  L  R  D  L  -

    GCTGCACCTGCTGGCATTCTCCAAATCCTGCTCCCTGCCGCAGACCTCAGGTCTTCAGAA
309 -+---------+---------+---------+---------+---------+--------- 368
    CGACGTGGACGACCGTAAGAGGTTTAGGACGAGGGACGGCGTCTGGAGTCCAGAAGTCTT
     L  H  L  L  A  F  S  K  S  C  S  L  P  Q  T  S  G  L  Q  K  -

    ACCGGAATCCCTGGACGGGGTCCTGGAAGCATCCCTGTACAGCACCGAAGTTGTTGCTCT
369 -+---------+---------+---------+---------+---------+--------- 428
    TGGCCTTAGGGACCTGCCCCAGGACCTTCGTAGGGACATGTCGTGGCTTCAACAACGAGA
     P  E  S  L  D  G  V  L  E  A  S  L  Y  S  T  E  V  V  A  L  -

    GTCCCGTCTGCAGGGTTCCCTTCAGGACATCCTTCAGCAGCTGGACGTTTCTCCGGAATG
429 -+---------+---------+---------+---------+---------+--------- 488
    CAGGGCAGACGTCCCAAGGGAAGTCCTGTAGGAAGTCGTCGACCTGCAAAGAGGCCTTAC
     S  R  L  Q  G  S  L  Q  D  I  L  Q  Q  L  D  V  S  P  E  C  -

    TTAATGGATCC
489 -+---------
    AATTACCTAGG
```

Herein, the first amino acid of the amino acid sequence for recombinant protein is referred to as +1, and is valine, and the amino acid at position -1 is methionine. The C-terminal amino acid is number 146 (cysteine).

The cloning of the murine OB DNA for expression in E. coli was done as follows. The DNA sequence was deduced from the published peptide sequence that appeared in Zhang et al., Nature 372:425-432 (1994). It was reverse translated using E. coli optimal codons. The terminal cloning sites were XbaI to BamHI. A ribosomal binding enhancer and a strong ribosomal binding site were included in front of the coding region. The duplex DNA sequence was synthesized using standard techniques. Correct clones were confirmed by demonstrating expression of the recombinant protein and presence of the correct OB DNA sequence in the resident plasmid.

Expression Vector and Host Strain

The plasmid expression vector used was pCFM1656, ATCC Accession No. 69576. The above DNA was ligated into the expression vector pCFM1656 which had been linearized with XbaI and BamHI and transformed into the E. coli host strain, FM5. E. coli FM5 cells were derived at Amgen Inc., Thousand Oaks, CA from E. coli K-12 strain (Bachmann, et al., Bacteriol. Rev. 40: 116-167 (1976)) and contain the integrated lambda phage repressor gene, cI_{857} (Sussman et al., C.R. Acad. Sci. 254: 1517-1579 (1962)). Vector production, cell transformation, and colony selection were performed by standard methods. E.g., Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2d Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Host cells were grown in LB media.

Fermentation Process A three-phase fermentation protocol was used known as a fed-batch process. Media compositions are set forth below.

Batch: A nitrogen and phosphate source were sterilized (by raising to 122 °C for 35 minutes, 18-20 psi) in the fermentation vessel (Biolafitte, 12 liter capacity). Upon cooling, carbon, magnesium, vitamin, and trace metal sources were added aseptically. An overnight culture of the above recombinant murine protein-producing bacteria (16 hours or more) of 500 mL (grown in LB broth) was added to the fermentor.

Feed I: Upon reaching between 4.0-6.0 OD_{600}, cultures were fed with Feed I. The glucose was fed at a limiting rate in order to control the growth rate (μ). An automated system (called the Distributive Control System) was instructed to control the growth rate to 0.15 generations per hour.

Feed II: When the OD_{600} had reached 30, culture temperature was slowly increased to 42°C and the feed was changed to Feed II, below. The fermentation was then allowed to continue for 10 hours with sampling every 2 hours. After 10 hours, the contents of the fermentor was chilled to below 20°C and harvested by centrifugation.

Media Composition:

Batch:	10 g/L	Yeast extract
	5.25 g/L	$(NH_4)_2SO_4$
	3.5 g/L	K_2HPO_4
	4.0 g/L	KH_2PO_4
	5.0 g/L	Glucose
	1.0 g/L	$MgSO_4 \cdot 7H_2O$
	2.0 mL/L	Vitamin Solution
	2.0 mL/L	Trace Metal Solution
	1.0 mL/L	P2000 Antifoam
Feed I:	50 g/L	Bacto-tryptone
	50 g/L	Yeast extract
	450 g/L	Glucose
	8.75 g/L	$MgSO_4 \cdot 7H_2O$
	10 mL/L	Vitamin Solution
	10 mL/L	Trace Metal Solution
Feed II:	200 g/L	Bacto-tryptone
	100 g/L	Yeast extract
	110 g/L	Glucose

Vitamin Solution (Batch and Feed I):

0.5 g Biotin, 0.4 g Folic acid, and 4.2 g riboflavin, were dissolved in 450 mls H_2O and 3 mls 10 N NaOH, and brought to 500 mls in H_2O. 14 g pyridoxine-HCl and 61 g niacin were dissolved 150 ml H_2O and 50 ml 10 N NaOH, and brought to 250 ml in H_2O. 54 g pantothenic acid was dissolved in 200 ml H_2O, and brought to 250 ml. The three solutions were combined and brought to 10 liters total volume.

Trace Metal Solution (Batch and Feed I):
Ferric Chloride ($FeCl_3 \cdot 6H_2O$): 27 g/L
Zinc Chloride ($ZnCl_2 \cdot 4H_2O$): 2 g/L
Cobalt Chloride ($CoCl_2 \cdot 6H_2O$): 2 g/L
Sodium Molybdate ($NaMoO_4 \cdot 2H_2O$): 2 g/L
Calcium Chloride ($CaCl_2 \cdot 2H_2O$): 1 g/L
Cupric Sulfate ($CuSO_4 \cdot 5H_2O$): 1.9 g/L
Boric Acid (H_3BO_3): 0.5 g/L
Manganese Chloride ($MnCl_2 \cdot 4H_2O$): 1.6 g/L
Sodium Citrate dihydrate: 73.5 g/L

<u>Purification Process for Murine OB Protein</u>

Purification was accomplished by the following steps (unless otherwise noted, the following steps were performed at 4°C):

1. Cell paste. <u>E</u>. <u>coli</u> cell paste was suspended in 5 times volume of 7 mM of EDTA, pH 7.0. The cells in the EDTA were further broken by two passes through a microfluidizer. The broken cells were centrifuged at 4.2 K rpm for 1 hour in a Beckman J6-B centrifuge with a JS-4.2 rotor.

2. Inclusion body wash #1. The supernatant from above was removed, and the pellet was resuspended with 5 times volume of 7 mM EDTA, pH 7.0, and homogenized. This mixture was centrifuged as in step 1.

3. Inclusion body wash #2. The supernatant from above was removed, and the pellet was resuspended in ten times volume of 20 mM tris, pH 8.5, 10 mM DTT, and 1% deoxycholate, and homogenized. This mixture was centrifuged as in step 1.

4. Inclusion body wash #3. The supernatant from above was removed and the pellet was resuspended in ten times volume of distilled water, and homogenized. This mixture was centrifuged as in step 1.

5. Refolding. The pellet was refolded with 15 volumes of 10 mM HEPES, pH 8.5, 1% sodium sarcosine (N-lauroyl sarcosine), at room temperature. After 60 minutes, the solution is made to be 60 μM copper sulfate, and then stirred overnight.

6. Removal of sarcosine. The refolding mixture was diluted with 5 volumes of 10 mM tris buffer, pH 7.5, and centrifuged as in step 1. The supernatant was collected, and mixed with agitation for one hour with Dowex® 1-X4 resin (Dow Chemical Co., Midland MI), 20-50 mesh, chloride form, at 0.066% total volume of diluted refolding mix. See WO 89/10932 at page 26 for more information on Dowex®. This mixture was poured into a column and the eluant was collected. Removal of sarcosine was ascertained by reverse phase HPLC.

7. Acid precipitation. The eluant from the previous step was collected, and pH adjusted to pH 5.5, and incubated for 30 minutes at room temperature. This mixture was centrifuged as in step 1.

8. Cation exchange chromatography. The pH of the supernatant from the previous step was adjusted to pH 4.2, and loaded on CM Sepharose Fast Flow (at 7% volume). 20 column volumes of salt gradient were done at 20 mM NaOAC, pH 4.2, 0 M to 1.0 M NaCl.

9. Hydrophobic interaction chromatography. The CM Sepharose pool of peak fractions (ascertained from ultraviolet absorbance) from the above step was made to be 0.2 M ammonium sulfate. A 20 column volume reverse salt gradient was done at 5 mM NaOAC, pH 4.2, with .4 M to 0 M ammonium sulfate. This material was concentrated and diafiltered into PBS.

Results

Presented below are the percent (%) differences from baseline weight in C57Bl6J mice (8 weeks old) :

Table 1: Weight Loss Upon Continuous Infusion

Time (days)	Vehicle (PBS)	Recombinant OB protein
Days 1-2	3.24 +/- 1.13	1.68 +/- 1.4
Days 3-4	4.3 +/- .97	-2.12 +/- .79
Days 5-6	4.64 +/- .96	-4.62 +/- 1.3

As can be seen, at the end of a 6 day continuous infusion regime, animals receiving the OB protein lost over 4% of their body weight, as compared to baseline. This is a substantially more rapid weight loss than has been observed with intraperitoneal (i.p.) injection. Weight loss at the end of a 32-day injection period, in wild type (normal) mice, with daily i.p. injections of recombinant murine OB protein at a 10 mg/kg dose was 2.6%, and had not been more than 4% at any time during the dosing schedule (data not shown). The present data indicate that with continuous infusion, a 20-fold lower dosage (0.5 mg/kg vs. 10 mg/kg) achieves more weight loss in a shorter time period.

The results seen here are statistically significant, e.g., -4.62% with $p < .0001$.

EXAMPLE 2: <u>Dose Response Studies</u>

An additional study demonstrated that there was a dose response to continuous administration of OB protein. In this study, non-obese, CD-1 mice, weighing 35-40 g were administered recombinant murine OB protein using methods similar to the above example. The results are set forth in Table 2, below, (with % body weight lost as compared to baseline, measured as above):

Table 2: Dose Response With Continuous Administration

Dose	Time	% Reduction in body weight
0.03 mg/kg/day	Day 2	3.5
1 mg/kg/day	Day 2	7.5
1 mg/kg/day	Day 4	14

As can be seen, increasing the dose from 0.03 mg/kg/day to 1 mg/kg/day increased the weight lost from 3.5% to 7.5%. It is also noteworthy that at day 4, the 1 mg/kg/day dosage resulted in a 14% reduction in body weight.

EXAMPLE 3: <u>Cloning and Expression of a Recombinant Human Methionyl OB Protein</u>

This example provides compositions and methods for preparation of a recombinant human version of the OB protein.

The human version of the OB DNA was constructed from the murine OB DNA, as in Example 1, above, by replacing the region between the MluI and BamHI sites with duplex DNA (made from synthetic oligonucleotides) in which 20 codon substitutions had been designed. The MluI site is shown

under the solid line in the sequence below. This DNA was put into the pCFM1656 vector (ATCC Accession No. 69576), in the same fashion as the recombinant murine protein, as described above. Herein, the first amino acid of the amino acid sequence for recombinant human protein below is referred to as +1, and is valine, and the amino acid at position -1 is methionine. The C-terminal amino acid is number 146 (cysteine).

<u>Recombinant human met OB (Double Stranded) DNA and amino acid sequence</u> (Seq. ID. Nos. 3 and 4)

```
      CATATGGTACCGATCCAGAAAGTTCAGGACGACACCAAAACCTTAATTAAAACGATCGTT
    1 ---------+---------+---------+---------+---------+---------+ 60
      GTATACCATGGCTAGGTCTTTCAAGTCCTGCTGTGGTTTTGGAATTAATTTTGCTAGCAA
        M  V  P  I  Q  K  V  Q  D  D  T  K  T  L  I  K  T  I  V   -

      ACGCGTATCAACGACATCAGTCACACCCAGTCGGTGAGCTCTAAACAGCGTGTTACAGGC
   61 ---------+---------+---------+---------+---------+---------+ 120
      TGCGCATAGTTGCTGTAGTCAGTGTGGGTCAGCCACTCGAGATTTGTCGCACAATGTCCG

      T  R  I  N  D  I  S  H  T  Q  S  V  S  S  K  Q  R  V  T  G   -

      CTGGACTTCATCCCGGGTCTGCACCCGATCCTGACCTTGTCCAAAATGGACCAGACCCTG
  121 ---------+---------+---------+---------+---------+---------+ 180
      GACCTGAAGTAGGGCCCAGACGTGGGCTAGGACTGGAACAGGTTTTACCTGGTCTGGGAC

      L  D  F  I  P  G  L  H  P  I  L  T  L  S  K  M  D  Q  T  L   -

      GCTGTATACCAGCAGATCTTAACCTCCATGCCGTCCCGTAACGTTCTTCAGATCTCTAAC
  181 ---------+---------+---------+---------+---------+---------+ 240
      CGACATATGGTCGTCTAGAATTGGAGGTACGGCAGGGCATTGCAAGAAGTCTAGAGATTG

      A  V  Y  Q  Q  I  L  T  S  M  P  S  R  N  V  L  Q  I  S  N   -

      GACCTCGAGAACCTTCGCGACCTGCTGCACGTGCTGGCATTCTCCAAATCCTGCCACCTG
  241 ---------+---------+---------+---------+---------+---------+ 300
      CTGGAGCTCTTGGAAGCGCTGGACGACGTGCACGACCGTAAGAGGTTTAGGACGGTGGAC

      D  L  E  N  L  R  D  L  L  H  V  L  A  F  S  K  S  C  H  L   -

      CCATGGGCTTCAGGTCTTGAGACTCTGGACTCTCTGGGCGGGGTCCTGGAAGCATCCGGT
  301 ---------+---------+---------+---------+---------+---------+ 360
      GGTACCCGAAGTCCAGAACTCTGAGACCTGAGAGACCCGCCCCAGGACCTTCGTAGGCCA

      P  W  A  S  G  L  E  T  L  D  S  L  G  G  V  L  E  A  S  G   -

      TACAGCACCGAAGTTGTTGCTCTGTCCCGTCTGCAGGGTTCCCTTCAGGACATGCTTTGG
  361 ---------+---------+---------+---------+---------+---------+ 420
      ATGTCGTGGCTTCAACAACGAGACAGGGCAGACGTCCCAAGGGAAGTCCTGTACGAAACC

      Y  S  T  E  V  V  A  L  S  R  L  Q  G  S  L  Q  D  M  L  W   -

      CAGCTGGACCTGTCTCCGGGTTGTTAATGGATCC
  421 ---------+---------+---------+---- 454
      GTCGACCTGGACAGAGGCCCAACAATTACCTAGG

      Q  L  D  L  S  P  G  C  *
```

<u>Fermentation</u>: Fermentation of the above host cells to produce recombinant human OB protein was accomplished using the conditions and compositions as described above

for recombinant murine material. The results were analyzed for yield (grams ob DNA product/liter of fermentation broth), prior to purification of the recombinant human OB material. (Minor amounts of bacterial protein were present.) Bacterial expression was also calculated.

Table 3: Analysis of Human OB Protein Expression

Timepoint	OD (@600 nm)	Yield (g/L)	Expression (mg/OD·L)
Ind. + 2 hours.	47	1.91	41
Ind. + 4 hours.	79	9.48	120
Ind. + 6 hours.	95	13.01	137
Ind. + 8 hours.	94	13.24	141
Ind. + 10 hours.	98	14.65	149

abbreviations: Ind. + __ hours means the hours after induction of protein expression, as described in Example I for the recombinant murine material using pCFM1656

OD: optical density, as measured by spectrophotometer milligrams per OD unit per liter

mg/OD·L: expression in terms of milligrams of protein per OD unit per liter.

g/L: grams protein/liter fermentation broth

Purification of the recombinant human OB protein: Recombinant human protein may be purified using methods similar to those used for purification of recombinant murine protein, as in Example 1, above. For preparation of recombinant human OB protein, step 8 was performed by adjusting the pH of the supernatant from step 7 to pH 5.0, and loading this onto a CM Sepharose fast flow column. The 20 column volume salt gradient was performed at 20 mM NaOAC, pH 5.5, 0M to 0.5 M NaCl. Step 9 was performed by diluting the CM Sepharose pool four fold with water, and adjusting the pH to 7.5. This mixture was made to 0.7 M ammonium sulfate. Twenty column volume reverse salt gradient was done at 5 mM NaOAC, pH 5.5, 0.2 M to 0M ammonium sulfate. Otherwise, the above steps were identical.

While the present invention has been described in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

CLAIMS

1. A method of treating excess weight in a mammal by continuous administration of 1 mg protein/kg body weight/day or less of an OB protein selected from the group consisting of:

(a) recombinant methionyl murine OB protein (SEQ. ID. No. 2);

(b) recombinant methionyl human OB protein (SEQ ID No. 1);

(c) the protein of (a) or (b) lacking the methionyl residue at position -1;

(d) the protein of (a), (b) or (c) lacking a glutamine at position 28; and

(e) a chemically modified derivative of (a), (b),(c) or (d).

2. A method of claim 1 wherein the chemically modified derivative is a pegylated derivative.

3. A method of claim 2 wherein the pegylated derivative is N-terminally pegylated.

4. A method of claim 1 wherein said continuous administration is accomplished by osmotic pump.

5. A DNA sequence according to SEQ ID No. 1.

6. A vector containing a DNA sequence according to claim 5.

7. A vector of claim 6 wherein said vector is pCFM1656.

8. A DNA sequence according to SEQ ID No. 3.

9. A vector containing a DNA sequence according to claim 8.

10. A vector according to claim 9 wherein said vector is pCFM1656.

11. A method of refolding partially purified OB protein in a solution obtained from inclusion bodies, said partially purified OB protein selected from the group consisting of:

(a) recombinant methionyl murine OB protein (SEQ. ID. No. 2);

(b) recombinant methionyl human OB protein (SEQ ID No. 1);

(c) the protein of (a) or (b) lacking the methionyl residue at position -1;

wherein said refolding is accomplished using N-lauroyl sarcosine.

12. A method of claim 11 wherein said sarcosine is used at a concentration of 0.5% - 2.0% weight per volume of solution.

ABSTRACT

The present invention provides methods and compositions for treating excess weight by administering OB protein in a form for constant supply, at a dosage of less than or equal to about 1 mg protein/kg body weight/day. Compositions and methods used for production of recombinant murine and human OB protein are also provided. Compositions and methods for preparing recombinant murine methionyl OB protein and recombinant human methionyl OB protein, including DNA sequences, vectors, host cells, methods of fermentation, and methods of purification are provided herein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Pelleymounter, Mary Ann
Hecht, Randy I
Mann, Michael B

(ii) TITLE OF INVENTION: OB PROTEIN COMPOSITIONS AND METHODS

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Amgen Inc.
(B) STREET: 1840 Dehavilland Drive
(C) CITY: Thousand Oaks
(D) STATE: California
(E) COUNTRY: U.S.A.
(F) ZIP: 91230-1789

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/474,833
(B) FILING DATE: 07-JUN-1995
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Pessin, Karol M.
(C) REFERENCE/DOCKET NUMBER: A-345

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 491 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
TCTAGATTTG AGTTTTAACT TTTAGAAGGA GGAATAACAT ATGGTACCGA TCCAGAAAGT     60

TCAGGACGAC ACCAAAACCT TAATTAAAAC GATCGTTACG CGTATCAACG ACATCAGTCA    120

CACCCAGTCG GTCTCCGCTA AACAGCGTGT TACCGGTCTG GACTTCATCC CGGGTCTGCA    180

CCCGATCCTA AGCTTGTCCA AAATGGACCA GACCCTGGCT GTATACCAGC AGGTGTTAAC    240

CTCCCTGCCG TCCCAGAACG TTCTTCAGAT CGCTAACGAC CTCGAGAACC TTCGCGACCT    300

GCTGCACCTG CTGGCATTCT CCAAATCCTG CTCCCTGCCG CAGACCTCAG GTCTTCAGAA    360
```

ACCGGAATCC CTGGACGGGG TCCTGGAAGC ATCCCTGTAC AGCACCGAAG TTGTTGCTCT 420

GTCCCGTCTG CAGGGTTCCC TTCAGGACAT CCTTCAGCAG CTGGACGTTT CTCCGGAATG 480

TTAATGGATC C 491

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 491 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGATCTAAAC TCAAAATTGA AAATCTTCCT CCTTATTGTA TACCATGGCT AGGTCTTTCA 60

AGTCCTGCTG TGGTTTTGGA ATTAATTTTG CTAGCAATGC GCATAGTTGC TGTAGTCAGT 120

GTGGGTCAGC CAGAGGCGAT TTGTCGCACA ATGGCCAGAC CTGAAGTAGG GCCCAGACGT 180

GGGCTAGGAT TCGAACAGGT TTTACCTGGT CTGGGACCGA CATATGGTCG TCCACAATTG 240

GAGGGACGGC AGGGTCTTGC AAGAAGTCTA GCGATTGCTG GAGCTCTTGG AAGCGCTGGA 300

CGACGTGGAC GACCGTAAGA GGTTTAGGAC GAGGGACGGC GTCTGGAGTC CAGAAGTCTT 360

TGGCCTTAGG GACCTGCCCC AGGACCTTCG TAGGGACATG TCGTGGCTTC AACAACGAGA 420

CAGGGCAGAC GTCCCAAGGG AAGTCCTGTA GGAAGTCGTC GACCTGCAAA GAGGCCTTAC 480

AATTACCTAG G 491

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 147 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Met Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys Thr Leu Ile Lys
1               5                   10                  15

Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr Gln Ser Val Ser
            20                  25                  30

Ala Lys Gln Arg Val Thr Gly Leu Asp Phe Ile Pro Gly Leu His Pro
        35                  40                  45

Ile Leu Ser Leu Ser Lys Met Asp Gln Thr Leu Ala Val Tyr Gln Gln
```

```
    50                  55                  60

Val Leu Thr Ser Leu Pro Ser Gln Asn Val Leu Gln Ile Ala Asn Asp
65                  70                  75                  80

Leu Glu Asn Leu Arg Asp Leu Leu His Leu Leu Ala Phe Ser Lys Ser
                85                  90                  95

Cys Ser Leu Pro Gln Thr Ser Gly Leu Gln Lys Pro Glu Ser Leu Asp
            100                 105                 110

Gly Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val Val Ala Leu Ser
        115                 120                 125

Arg Leu Gln Gly Ser Leu Gln Asp Ile Leu Gln Gln Leu Asp Val Ser
    130                 135                 140

Pro Glu Cys
145
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 454 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
CATATGGTAC CGATCCAGAA AGTTCAGGAC GACACCAAAA CCTTAATTAA AACGATCGTT    60

ACGCGTATCA ACGACATCAG TCACACCCAG TCGGTGAGCT CTAAACAGCG TGTTACAGGC   120

CTGGACTTCA TCCCGGGTCT GCACCCGATC CTGACCTTGT CCAAAATGGA CCAGACCCTG   180

GCTGTATACC AGCAGATCTT AACCTCCATG CCGTCCCGTA ACGTTCTTCA GATCTCTAAC   240

GACCTCGAGA ACCTTCGCGA CCTGCTGCAC GTGCTGGCAT TCTCCAAATC CTGCCACCTG   300

CCATGGGCTT CAGGTCTTGA GACTCTGGAC TCTCTGGGCG GGGTCCTGGA AGCATCCGGT   360

TACAGCACCG AAGTTGTTGC TCTGTCCCGT CTGCAGGGTT CCCTTCAGGA CATGCTTTGG   420

CAGCTGGACC TGTCTCCGGG TTGTTAATGG ATCC                                454
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 454 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
GTATACCATG GCTAGGTCTT TCAAGTCCTG CTGTGGTTTT GGAATTAATT TTGCTAGCAA     60
TGCGCATAGT TGCTGTAGTC AGTGTGGGTC AGCCACTCGA GATTTGTCGC ACAATGTCCG    120
GACCTGAAGT AGGGCCCAGA CGTGGGCTAG GACTGGAACA GGTTTTACCT GGTCTGGGAC    180
CGACATATGG TCGTCTAGAA TTGGAGGTAC GGCAGGGCAT TGCAAGAAGT CTAGAGATTG    240
CTGGAGCTCT TGGAAGCGCT GGACGACGTG CACGACCGTA AGAGGTTTAG GACGGTGGAC    300
GGTACCCGAA GTCCAGAACT CTGAGACCTG AGAGACCCGC CCCAGGACCT TCGTAGGCCA    360
ATGTCGTGGC TTCAACAACG AGACAGGGCA GACGTCCCAA GGGAAGTCCT GTACGAAACC    420
GTCGACCTGG ACAGAGGCCC AACAATTACC TAGG                                454
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 147 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys Thr Leu Ile Lys
1               5                   10                  15

Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr Gln Ser Val Ser
            20                  25                  30

Ser Lys Gln Arg Val Thr Gly Leu Asp Phe Ile Pro Gly Leu His Pro
        35                  40                  45

Ile Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala Val Tyr Gln Gln
    50                  55                  60

Ile Leu Thr Ser Met Pro Ser Arg Asn Val Leu Gln Ile Ser Asn Asp
65                  70                  75                  80

Leu Glu Asn Leu Arg Asp Leu Leu His Val Leu Ala Phe Ser Lys Ser
                85                  90                  95

Cys His Leu Pro Trp Ala Ser Gly Leu Glu Thr Leu Asp Ser Leu Gly
            100                 105                 110

Gly Val Leu Glu Ala Ser Gly Tyr Ser Thr Glu Val Val Ala Leu Ser
        115                 120                 125

Arg Leu Gln Gly Ser Leu Gln Asp Met Leu Trp Gln Leu Asp Leu Ser
    130                 135                 140

Pro Gly Cys
145
```

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first, and sole inventor (if only one name is listed below) or a joint inventor (if plural names are listed below) of the invention entitled

OB PROTEIN COMPOSITIONS AND METHODS

which is described and claimed in the specification which:

[X] is attached hereto.

[ ] was filed on ____________________
as Application Serial No.: ____________
and was amended on ____________ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

Power of Attorney: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

Ron K. Levy, Registration No.: 31,539, Steven M. Odre, Registration No.: 29,094, and Karol M. Pessin, Registration No. 34,899, said attorney(s)/agent(s) to have in addition full power of revocation, including the power to revoke any power herein granted.

Please send all future correspondence to:

U.S. Patent Operations/KMP
M/S 10-1-B
AMGEN INC.
Amgen Center
1840 Dehavilland Drive
Thousand Oaks, California 91320-1789

Direct Telephone Calls To:

Attorney/Agent's Name
Attorney/Agent for Applicant(s)
Registration No.: 34,899
Phone: (805) 447-2425
Date: June 5, 1995

"Express Mail" mail labeling number TB 530 346 498 US
Date of Deposit June 7, 1995
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D. C. 20231

Paul F Fehlner
Printed Name

Paul F Fehlner 6/7/95
Signature

DECLARATION AND POWER OF ATTORNEY (cont'd)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Full Name of Sole or First Inventor: MARY ANN PELLEYMOUNTER

Inventor's Signature: Mary Ann Pelleymounter Date: 6/5/95

Residence: 1909 Hillcrest Drive

Post Office Address: Thousand Oaks, CA 91320

Citizenship: United States

Full Name of Second Joint Inventor, if Any: RANDY IRA HECHT

Inventor's Signature: Randy Ira Hecht Date: 6/5/95

Residence: 3316 Silver Spur Court

Post Office Address: Thousand Oaks, CA 91360

Citizenship: United States

Full Name of Third Joint Inventor, if Any: MICHAEL BENJAMIN MANN

Inventor's Signature: Michael Benjamin Mann Date: 6/5/95

Residence: 1506 Rugby Circle

Post Office Address: Thousand Oaks, CA 91360

Citizenship: United States

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Pelleymounter et al.

Serial No.: 08/920,608

Group Art Unit No.: 1812

Filed: August 27, 1997

Examiner: G. Draper

For: OB Protein Compositions and Methods

Docket No.: A-345A

ASSOCIATE POWER OF ATTORNEY

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Please recognize Joan D. Eggert, Registration No.: 32,980, as associate attorney in this application, with full power to prosecute the application, to make alterations and amendments therein, and to transact such other business in the Office in connection therewith as may be necessary.

Respectfully submitted,

Karol Pessin

Karol M. Pessin
Attorney for Applicants
Registration No.: 34,899
Phone: (805) 447-2193
Date: February 6, 1998

Please send all future correspondence to:

U.S. Patent Operations/JDE
M/S 10-1-B
AMGEN INC.
Amgen Center
One Amgen Center Drive
Thousand Oaks, California 91320-1789

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231, on the date appearing below

February 6, 1998
Date

Signature